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Salinity is a key factor driving the nitrogen cycling in the mangrove sediment

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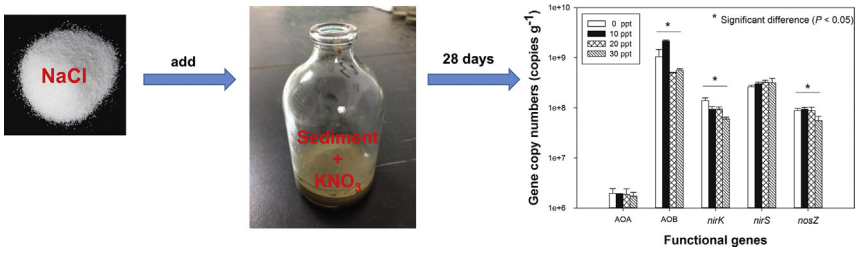
HIGHLIGHTS

- Salinity elevation decreased both the activity and abundance of denitrifiers.
- The nitrifiers (AOB) were most abundant under the intermediate salinity conditions.
- Salinity significantly shaped the nitrifying and denitrifying community structures.
- These findings may increase concern regarding threats of salinity intrusion.

GRAPHICAL ABSTRACT



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ABSTRACT

Coastal ecosystems are hotspots for nitrogen cycling, and specifically for nitrogen removal from water and sediment through the coupled nitrification-denitrification process. Salinity is globally important in structuring bacterial and archaeal communities, but the association between salinity and microbially-mediated nitrification and denitrification remains unclear. The denitrification activity and composition and structure of microbial nitrifiers and denitrifiers were characterized across a gradient of manipulated salinity (0, 10, 20 and 30 ppt) in a mangrove sediment. Salinity negatively correlated with both denitrifying activity and the abundance of *nirK* and *nosZ* denitrifying genes. Ammonia-oxidizing bacteria (AOB), which dominated nitrification, had significantly greater abundance at intermediate salinity (10 and 20 ppt). However, a positive correlation between ammonia concentration and salinity suggested that nitrifying activity might also be inhibited at higher salinity. The community structure of ammonia-oxidizing archaea (AOA) and bacteria (AOB), as well as *nirK*, *nirS* and *nosZ* denitrifying communities, were all significantly correlated with salinity. These changes were also associated with structural shifts in phylogeny. These findings provide a strong evidence that salinity is a key factor that influences the nitrogen transformations in coastal wetlands, indicating that salinity intrusion caused by climate change might have a broader impact on the coastal biospheres.

1. Introduction

Coastal wetlands such as mangroves dominate the tropical and subtropical coastlines, but are globally disappearing (Duke et al., 2007). These ecosystems are hotspots of nutrient cycling and play pivotal roles in nutrient transformation and availability (Fisher and Acreman, 2004). Recent efforts have been focused on determining how climate

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change may influence these cycling processes (Duke et al., 2007; Galloway et al., 2008; Gruber and Galloway, 2008), including the consequences of salinity intrusion resulting from groundwater abstraction, declining sediment loads and rising sea level, on nitrogen cycling (Bernhard et al., 2005; Bernhard et al., 2007; Franklin et al., 2017; Liu et al., 2017; Sheng et al., 2015; Zhou et al., 2017). Nitrification and denitrification are responsible for ammonia oxidation and nitrate reduction, respectively. The coupled nitrification-denitrification process drives the nitrogen removal in wetlands, thus preventing eutrophication (Fisher and Acreman, 2004; Vymazal, 2007).

Many efforts have focused on how nitrifying and denitrifying communities respond to the salinity elevation in coastal wetlands (Bernhard et al., 2005; Bernhard et al., 2010; Bernhard et al., 2007; Marton et al., 2012; Sheng et al., 2015; Xie et al., 2014; Zhang et al., 2015), yet the answer remains ambiguous. Ammonia oxidation, which is the first step of nitrification conducted by the ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) shows varying responses to salinity in different environments. Moderate salinity (10–20 ppt) is associated with an increased abundance of AOA and potential nitrification rates, while AOB either show no or negative correlation with increase salinity (Bernhard et al., 2005; Bernhard et al., 2010; Bernhard et al., 2007; Zhang et al., 2015). A study in the Douro River estuary observed increased nitrification rate with a salinity increase from 0 to 15 ppt (Magalhaes et al., 2005). Estuarine nitrifiers appear to grow optimally at 5–10 ppt, and are inhibited if soil salinity exceeds 10 ppt (Zhou et al., 2017). These studies suggest that optimum nitrification rate or nitrifier abundance normally occurs in middle salinity ranges although some varying results existed. Increasing salinity can impact nitrification directly by constraining the fitness of nitrifiers and indirectly by influencing the availability of oxygen which can further impact the soil respiration (Zhou et al., 2017). However, ammonia adsorption in sediment is also decreased with an increase in salinity, potentially contributing to increased ammonia efflux, which further stimulates nitrification (Seitzinger et al., 1991; Rysgaard et al., 1999).

Denitrification, which returns nitrogen back to the atmosphere as N_2O and N_2, also shows different relationships with salinity. It has been shown to be negatively associated with sediment salinity in the range of 0–36 ppt in certain estuaries (Giblin et al., 2010; Santoro et al., 2008), but also to have no association with salinities between 2 and 24 ppt in other places (Fear et al., 2005). Denitrifier abundance and potential have been associated with low salinities around 5 ppt (Franklin et al., 2017; Marton et al., 2012). Salinity can influence denitrification by altering the accessibility of organic substrates which are essential for heterotrophic bacteria (Franklin et al., 2017). The influence of increased salinity on nitrification can change the nitrate availability, which can also affect the denitrification (Giblin et al., 2010). Moreover, dissimilatory nitrate reduction to ammonium (DNRA) can compete for nitrate with denitrification since DNRA is favored over denitrification with high storage of sulfides which is always associated with high salinity conditions such as coastal and marine ecosystems (Giblin et al., 2013; Marchant et al., 2014). Salinity also exhibits a significant impact on soil bacterial and fungal community structure (Asghar et al., 2012; Behera et al., 2017; Chen et al., 2017; Mohamed and Martiny, 2011). However, only several studies have explored the association between salinity and nitrifying or denitrifying community structure (Bañeras et al., 2012; Bernhard et al., 2007; Franklin et al., 2017; Sahan and Muyzer, 2008; Xie et al., 2014).

The differences of the response to salinity in nitrogen cycling processes and the structure of the responsible microorganisms are to be expected because of the influence of un-measured environmental variables. It is hence essential to control environmental systems as much as possible. In this study, a laboratory incubation experiment was conducted to investigate the response of mangrove sediment nitrifying and denitrifying communities to different salinities (0, 10, 20 and 30 ppt). Denitrifying activity was measured as stimulated N_2O emission, and the abundance of nitrifiers (AOA and AOB) and denitrifiers (*nirK*, *nirS* and *nosZ*) were estimated by real-time quantitative PCR (qPCR). The nitrifying community composition and structure (AOA and AOB) was assessed by clone library, while high-throughput sequencing was performed for denitrifying communities (*nirK*, *nirS* and *nosZ*). This study aims to reveal the impact of salinity on both nitrifying and denitrifying processes and the potential influences of changes in coupled nitrification-denitrification on the nitrogen transformations and ecosystem functions.

2. Materials and methods

2.1. Incubation experiment

The surface sediment (0–50 cm) from an unvegetated area in the mangrove wetland located in the mudflat of Jiulong River estuary (24° 27′ N; 117° 54′ E) was collected as previously described (Wang et al., 2014). The original salinity of the sediment was around 15 ppt (parts-per-thousand). To accurately manipulate the incubations, sediment was air-dried, ground and sieved (<2 mm) before experimental setup. The pH and salinity of the treated sediment measured as sediment to water ratio of 1:5 (g/mL) were 7.06 and 3 ppt respectively. The concentrations of ammonium (NH_4^+) and nitrate and nitrite (NO_x^-) were 12.9 and 1.94 μg g^{-1} dry sediment, respectively. The dried sediment was kept for three months before the start of the incubation experiment. An aliquot of 10 g dry sediment was placed in 120-mL serum bottles and then 10 mL distilled water was added to each bottle. All the serum bottles were sealed with the wraps with tiny holes to let the air go through but prevent the water running off. Then these bottles were incubated at 25 °C in a dark and aerobic environment for one month to recover the microorganisms. After that, the different salinities were manipulated by adding solutions with different concentrations of NaCl. The incubations were categorized into 4 groups representing 4 salinity gradients. Each group received corresponding 5-mL NaCl solutions. The final salinity gradients were 0 ppt, 10 ppt, 20 ppt and 30 ppt as calculated by the added NaCl to the added water (15 mL in total for each bottle). In addition to NaCl, 300 μM KNO_3 was also added to each incubation to stimulate the nitrogen process. After adding the solutions, all the bottles were incubated at 25 °C in a dark and aerobic environment for another one month. Sediment samples were taken at 0, 7, 14, 21 and 28 days. For each sampling, two of three incubations of each salinity gradient were taken for DNA extraction and nutrient measurement, and for determining denitrifying activity. The used incubations were excluded from future sampling to avoid the unbalance of sample quantities. In total, 120 incubations were processed, including 60 each (4 salinity gradients × 5 time points × 3 replicates) for DNA extraction and denitrifying activity determination.

2.2. Denitrifying activity and nutrient measurement

The potential denitrifying activity was measured according to the C_2H_2 inhibition method. At each time point, three incubations of each salinity gradient were sealed with rubber stoppers and then evacuated and flushed with 99.999% helium three times. C_2H_2 was injected to a final composition of 10% (vol/vol). The sediments were then incubated at 25 °C on a magnetic stirring apparatus for 12 days. The headspace gases were analyzed at every 2, 4 or 12 h for the first two days, and then every 24 or 48 h for the next 10 days with a gas chromatographer equipped with an ECD detector as previously described (Molstad et al., 2007). The denitrifying activity was calculated by linear regression of accumulated N_2O per gram dry sediment *versus* time. The concentrations of NH_4^+ and NO_x^- were determined using 2 M KCl extracts with a 1:10 ratio of fresh sediment to KCl solution (g/mL). The extracts were further analyzed by FIA QC8500 continuous flow injection analyzer (LACHAT, USA).

2.3. DNA extraction and gene quantification

Total DNA was extracted from 0.5 g fresh sediment with the FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The purity and concentration of the extracted DNA were measured using UV–vis spectrophotometer ND-1000 (NanoDrop, USA). The extracted DNA was then stored at −20 °C for molecular experiments.

The primer pairs used for amplifying and quantifying the archaeal and the β-proteobacteria *amoA* genes, *nirS*, *nirK* and *nosZ* genes were described in previous studies (Wang et al., 2014; Wang et al., 2015). The conditions used for real-time quantitative PCR (qPCR) of these 5 genes were also described in the two previous studies, with the exception that reactions were conducted on a LightCycler 480II Real-Time PCR System (Roche, IN, USA). To evaluate the relative abundance of each gene, the abundances of bacterial and archaeal 16S rRNA genes were also quantified. The primer pairs and conditions used for 16S rRNA genes were described in a previous study (Wang et al., 2017). The conditions of qPCR and primers for all genes used in this study were summarized in Table S1. Standard curves of qPCR were obtained by serially diluting standard plasmids containing the target genes with known copy numbers. Negative controls without DNA template were included in each amplification. Inhibition was eliminated by highly diluting the DNA extracts. PCR efficiency above 90% was accepted.

2.4. Clone libraries, high-throughput sequencing and phylogenetic analysis

The DNA samples from day 28 were used for microbial community composition analysis. The community compositions of AOA and AOB were analyzed by cloning while the community compositions of *nirK*, *nirS* and *nosZ* were tested using high-throughput sequencing. The PCR mixture and reaction of AOA and AOB were described in the previous study (Wang et al., 2015). The PCR products of the 12 samples were used to construct the clone libraries and 50 clones for each library were selected for sequencing. In total, 599 clean sequences were used for clustering for both AOA and AOB. Similarity of 97% was used as the threshold for picking operational taxonomic unit (OTU) using Mothur v1.19 (Schloss et al., 2009).

For denitrifying genes, each reverse primer corresponding to a certain sample was tagged with a six-base barcode. Amplifications of *nirK*, *nirS* and *nosZ* were processed as previously described (Wang et al., 2014), but with the barcoded primers. PCR products were purified with the Universal DNA Purification Kit (TIANGEN, China). The purified product concentrations were then confirmed using the QuantiFluor dsDNA System (Promega, CA, USA). The barcoded PCR products were equally pooled, which were then precipitated by ethanol with sodium acetate and dissolved in sterilized Milli-Q water. The final DNA pool was sequenced on an Illumina MiSeq PE300 platform. The sequencing data were analyzed using QIIME v1.9.1 (Caporaso et al., 2010). Raw sequences were demultiplexed and low quality or ambiguous reads were removed. The frame-shift errors were checked using the HMM-FRAME algorithm (Zhang and Sun, 2011) together with Hidden Markov Models of different genes from the FunGene database (Fish et al., 2013). Sequences with frame-shit errors were discarded. In total, there were 401,963, 729,176 and 508,882 clean sequences for *nirK*, *nirS* and *nosZ*, respectively. Filtered sequences were then clustered into OTUs based on 97% similarity using the "pick_otu.py" function with USEARCH method. The singletons and chimeric sequences were removed during the OTU picking. After that, each sample was rarefied to equal sequence depth (the smallest number of the sequences among all samples) for downstream analyses.

The representative sequences of OTUs with a relative abundance above 1% were blasted against the GenBank nucleotide database and reference sequences were chosen as the highly similar sequences to each OTU. Then both the representative and reference sequences were deduced to amino acid to build the neighbor-joining phylogenetic trees with 1000 times bootstrap using MEGA v7.0 (Kumar et al., 2016).

All the sequences of AOA and AOB clones were submitted to GenBank with the accession numbers MF566141-MF566739 and MF566740-MF567338, respectively. All the sequences of *nirK*, *nirS* and *nosZ* were submitted to the European Nucleotide Archive of EMBL with the accession number PRJEB21903.

2.5. Statistical analysis

Analysis of variance (ANOVA) was employed to observe the significance of the salinity impact on the denitrifying activity, nutrient concentrations, gene abundances and OTU relative abundances. Data failed to meet the assumptions of ANOVA were log transformed or analyzed by the non-parametric Kruskal-Wallis test. The Tukey *post hoc* multiple comparisons were conducted to compare the differences between each two of the salinity gradients. Nonmetric multidimensional scaling (nMDS) based on the Bray-Curtis dissimilarity matrix was performed to determine the influence of salinity on the nitrifying and denitrifying community compositions. Analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were used to test the significance of the salinity impact on the microbial community structures. Pairwise Pearson correlations were conducted between denitrifying activity, NH_4^+ concentration, NO_x^- concentration and abundances of nitrifying and denitrifying genes. All the *P*-values were adjusted by false discovery rate (FDR) method for multiple comparisons and the null hypothesis was rejected while *P*-values was <0.05. All the statistical analyses were processed in R with *vegan*, *car* and *Hmisc* packages.

3. Results

3.1. Denitrifying activity and nutrient concentrations

The activity of denitrification was calculated as the average N_2O emission per day during a 12-day incubation after sampling (Fig. S1). The emission rate in sediment with greater salinity (20–30 ppt) was significantly lower compared to the lower salinity (0–10 ppt). One exception to this trend was on day 7 and 14 of the incubations, when emissions were significantly reduced in 10-ppt, when compared to the 0-ppt control (Fig. 1a). During the 28-day incubation, the ammonia was depleted in 0- and 10-ppt sediments on day 21 and 28, respectively (Fig. 1b). Ammonia concentrations were also significantly lower than that in 30-ppt samples on day 7 and 14. Although ammonia concentration in 20-ppt sediments showed an increase on day 14 and 21, it decreased dramatically on day 28. These results suggest the inhibition of ammonia consumption by high salinity. However, the NO_x^- concentration exhibited an opposite pattern. The concentration was significantly lower in both the 20- and 30-ppt samples on day 14, while the lowest NO_x^- concentration occurred in 20- and 30-ppt samples on day 21 and day 28, respectively (Fig. 1c). Despite that, the overall NO_x^- concentrations were not significantly different from day 0 to day 28. Correlation analysis demonstrated that the NH_4^+ and NO_x^- concentrations were negatively and positively associated with the average N_2O emission rate, respectively ($P < 0.05$, Fig. S2).

3.2. The abundances of nitrifying and denitrifying genes

The abundances of *amoA* genes for both AOA (Fig. 2a) and AOB (Fig. 2b) increased from day 0 to day 28. For AOA, the difference only occurred on day 7 that *amoA* gene abundance was significantly lower in high salinity samples (20 and 30 ppt) than in the low salinity samples (0 and 10 ppt) (Fig. 2a). Interestingly, the abundance of bacterial *amoA* gene was significantly higher in the intermediate salinity samples (10 or 20 ppt) compared to those in the low (0 ppt) or high (30 ppt) salinity samples (Fig. 2b), suggesting a different response to salinity

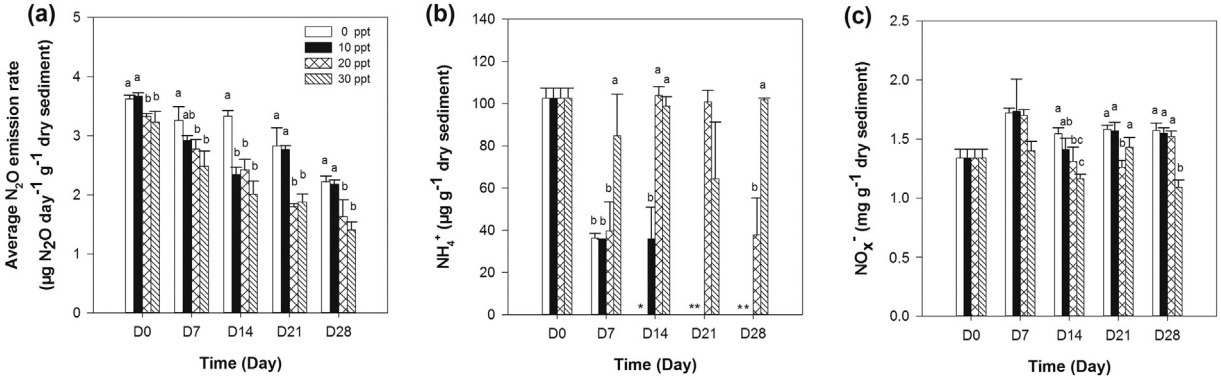

Fig. 1. Changes in the average N_2O emission rate (a), ammonia concentration (b), and nitrate and nitrite concentration (c) on day 0, day 7, day 14 and day 28. The different letters among samples at each time point indicate the significant difference ($P < 0.05$). The values are given as mean ± standard deviation (n = 3). *, undetected.

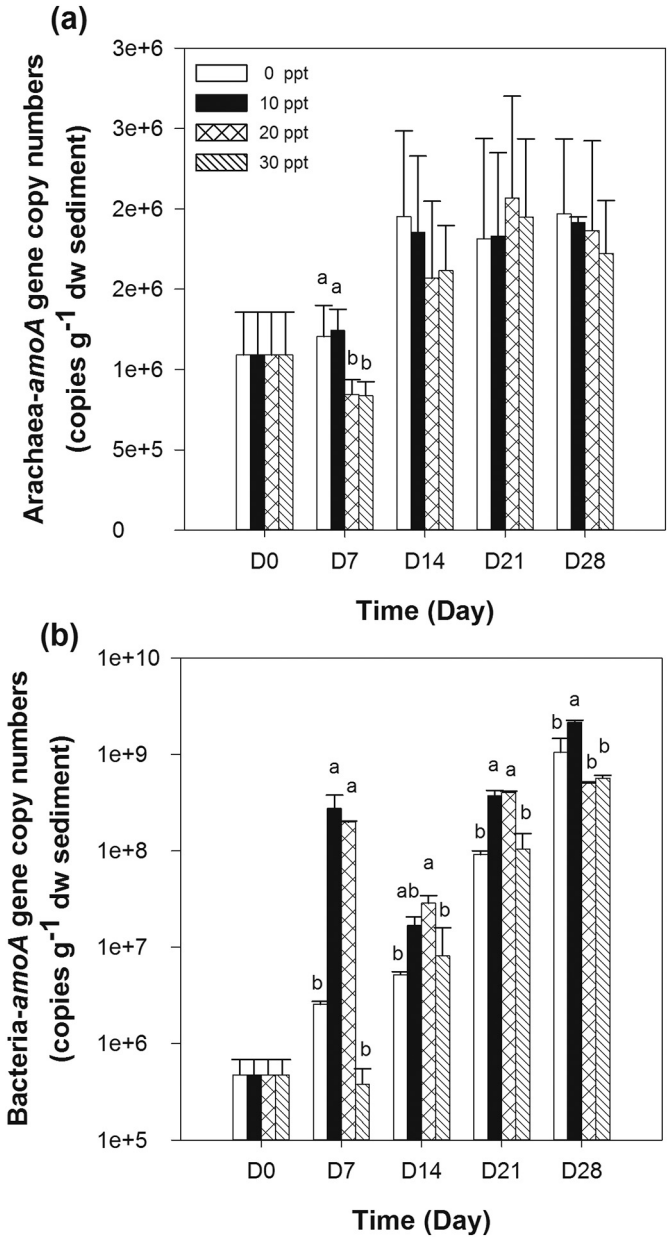

Fig. 2. Changes in the *amoA* gene abundance of AOA (a) and AOB (b) on day 0, day 7, day 14 and day 28. The different letters among samples at each time point indicate the significant difference ($P < 0.05$). The values are given as mean ± standard deviation (n = 3).

impact between AOB and AOA. Moreover, the AOB abundance was several orders of magnitude greater than AOA abundance, despite comparable abundance on day 0, suggesting that AOB dominated nitrification. The relative abundance calculated as the copy number ratio of functional gene to corresponding 16S rRNA gene was investigated (Fig. S3). The results of relative abundances of AOA and AOB showed a similar trend, with the exception that difference of AOA relative abundance also occurred on day 21 when the abundance was significantly lower in high salinity samples (20 and 30 ppt) compared with 0-ppt samples (Fig. S3).

Salinity had a limited influence on denitrifying gene abundance. Only on day 28 the lowest and highest *nirK* abundance was detected in 30-ppt and 0-ppt samples, respectively (Fig. 3a). While there was no significant change in *nirS* abundance (Fig. 3b), the *nosZ* abundance was significantly lower in 30-ppt samples compared to the other samples on day 28 (Fig. 3c). On day 28, the *nirK*/*nirS* ratio was significantly greater in 0-ppt samples compared to the other samples (Fig. 3d), while the *nir*/*nosZ* ratio was greatest in 30-ppt samples (Fig. 3e). The *nir*/*nosZ* ratio was positively and significantly correlated with denitrifying activity and NO_x^- concentration ($P < 0.05$, Fig. S2). The relative abundances of *nirK*, *nirS* and *nosZ* all showed a similar trend to the absolute abundances (Fig. S3).

3.3. The community compositions of nitrifiers and denitrifiers

In total, 53 and 26 OTUs were obtained for AOA and AOB communities, respectively. nMDS plots showed that both AOA and AOB communities were grouped according to different salinity gradients (Fig. 4a and b). Both the ANOSIM and PERMANOVA indicated that salinity significantly impacted AOA and AOB community composition ($P < 0.01$, Table 1). For denitrifying communities, 1607, 7259 and 6148 OTU were observed for *nirK*, *nirS* and *nosZ* communities, respectively. The *nirK* and *nosZ* communities of samples associated with different salinity gradients were well grouped while the *nirS* communities were less distinctly distributed (Fig. 4c, d and e). However, the *nirK*, *nirS* and *nosZ* community compositions were all significantly influenced by salinity as demonstrated by the ANOSIM and PERMANOVA results ($P < 0.05$, Table 1).

3.4. Phylogenetic analysis

To reduce the effect of rare OTUs on phylogenetic tree construction, we chose the OTUs with a relative abundance >1%. The distribution of abundant OTUs for different genes are displayed in Fig. S4. According to the phylogenetic tree for AOA (Fig. S5) and AOB (Fig. S6), the abundant OTUs could be grouped into 4 and 3 clusters, respectively. The AOA cluster I, which was associated with *Nitrososphaera* (Fig. S5), accounted for the largest proportion (74.0%) of AOA sequences. The relative abundance of AOA cluster I was slightly greater in the 0- and 10-

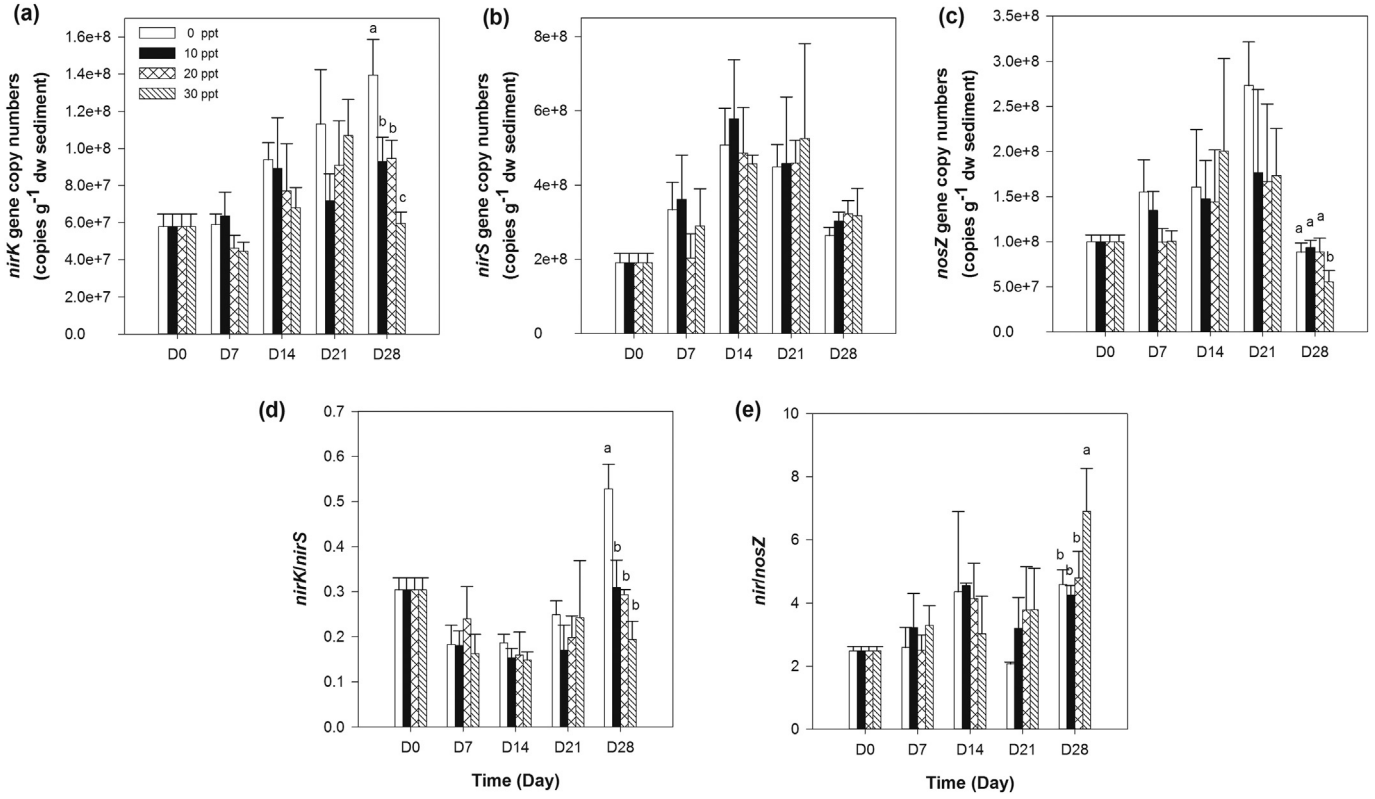

Fig. 3. Changes in the abundances of denitrifying genes, *nirK* (a), *nirS* (b) and *nosZ* (c), as well as the *nirK*/*nirS* (d) and *nir*/*nosZ* (e) ratios on day 0, day 7, day 14 and day 28. The different letters among samples at each time point indicate the significant difference ($P < 0.05$). The values are given as mean ± standard deviation (n = 3).

ppt samples compared to those in 20- and 30-ppt samples (Fig. 5a). The AOB cluster II which was affiliated with *Nitrosomonas* (Fig. S6) occupied 76.4% of the total AOB members. Both AOB cluster I and II dominated 0- and 10-ppt samples while only cluster II flourished in 20- and 30-ppt samples (Fig. 5b), which suggested that high salinity inhibited the cluster I of AOB which was associated with *Nitrosospira* (Fig. S6).

Based on the phylogenetic analyses of denitrifying communities, the *nirK* (Fig. S7), *nirS* (Fig. S8) and *nosZ* (Fig. S9) sequences can be grouped into 3, 10 and 7 clusters, respectively. For *nirK*, cluster I was the dominant cluster accounting for 58.4% of the total sequences. This cluster was significantly greater in the high salinity (20 and 30 ppt) samples than in the low salinity (0 and 10 ppt) samples (Fig. 5c, TukeyHSD $P < 0.05$), suggesting preference of high salinity condition of this cluster. However, *nirK* cluster I cannot be assigned to a known taxon (Fig. S7). For *nirS*, only cluster V and VII showed a slightly different abundance among different salinity gradients (Fig. 5d). For *nosZ*, cluster I and cluster III were the dominant clusters, with a proportion of 10.8% and 20.2%, respectively. The abundance of cluster III which might be associated with *Thiobacillus* (Fig. S9) was significantly lower in 30-ppt samples compared to 0-ppt samples (Fig. 5e, TukeyHSD $P < 0.05$).

4. Discussion

The denitrifying activity, abundance of nitrifiers and denitrifiers, and their community structures were investigated in mangrove sediments treated with different salinities. Salinity exhibited a significant influence on the nitrogen cycling processes. Increase of salinity from 0 to 30 ppt decreased both denitrification activity and the abundance of denitrifiers, while the nitrifiers were most abundant under the intermediate salinity conditions. The community composition of nitrifiers and denitrifiers was significantly influenced by salinity. Although this study is based on the manipulated sediment recovered from the dried sediment, the recovered microbial communities were originally from the sediment in the field, and thus partly representing the *in-situ* condition. These findings suggest that salinity intrusion in coastal areas might interrupt the balance of nutrient cycles, and could negatively contribute to ecosystem functions.

Salinity exhibited a limited influence on AOA abundance in this study, which is different from previous studies showing that AOA was the most abundant at intermediate salinity (10–30 ppt) (Bernhard et al., 2010; Zhang et al., 2015). However, AOB played a dominant role in nitrification in this study since the AOB abundance outcompeted AOA abundance. The relative importance of AOA and AOB for nitrification in saline environments remains controversial. Consistent with this study, the AOB abundance was greater than AOA in sediments from Douro River estuary despite of the varied salinities ranging from 1.5 to 26.8 ppt (Magalhães et al., 2009). In some estuaries, it was found that AOA predominated the nitrification in the regions with low salinity while AOB were more abundant than AOA in higher salinity regions (Mosier and Francis, 2008; Santoro et al., 2008). In contrast, AOA abundance was greater than AOB at all the salinity levels in Plum Island Sound estuary (Bernhard et al., 2010). These divergent results indicate that other factors influence the abundance of AOA and AOB. It is suggested that the dominance of AOB over AOA normally occurs in mangrove sediments as the slightly acidic or neutral condition and the available ammonia might favor the growth of AOB (Li et al., 2011; Li and Gu, 2013; Wang et al., 2015). Moreover, we found that AOB abundance was greater in the 10- or 20-ppt sediments during the 28-day incubation. This finding supports a previous meta-analysis showing that nitrification can be promoted within a certain range of the salinity and inhibited when salinity exceeds this range (Zhou et al., 2017). For this study, the intermediate salinities (10 and 20 ppt) were considered as the optimal condition for the ammonia oxidizers, which might also result from the long-term adaption of the microbes to the local habitats where the sediment salinity normally shifted between 10 and 20 ppt (Wang et al., 2016). We observed that NH_4^+ concentration was significantly higher in the 20- and 30-ppt samples, suggesting the less consumption of NH_4^+ which might be caused by the reduction of

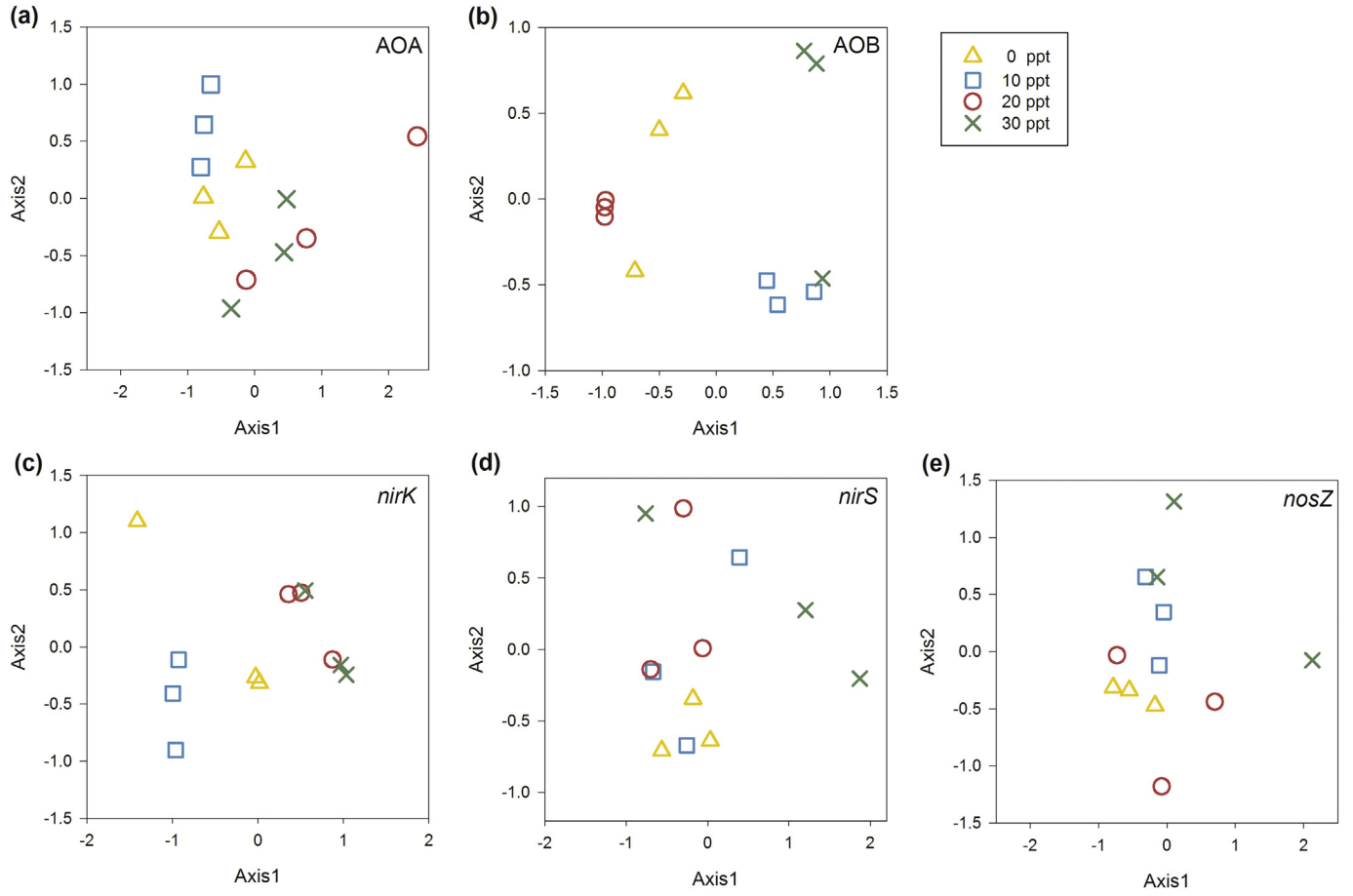

Fig. 4. Nonmetric multidimensional scaling (nMDS) plots of AOA (a), AOB (b), *nirK* (c), *nirS* (d) and *nosZ* (e) based on the Bray-Curtis dissimilarities.

nitrification activity. However, no significant correlation was found between NH_4^+ concentration and AOB abundance. This may be due to the enhanced DNRA activity as DNRA is favored under the higher salinity condition (Giblin et al., 2013; Marchant et al., 2014), which might further offset the consumed NH_4^+.

In addition to the abundance of nitrifiers, salinity exhibited a significant impact on both AOA and AOB community structure. These results support previous studies showing that sediment salinity was the main factor controlling the distribution of AOA or/and AOB (Sahan and Muyzer, 2008; Zhang et al., 2015). The genus *Nitrososphaera* dominated AOA community in all samples, but was slightly restricted at high salinity levels in this study. However, as only one *Nitrososphaera* isolate has been identified (Tourna et al., 2011), little can be said about its potential interaction. For AOB, we found that both the high and low salinities favored *Nitrosomonas* while *Nitrosospira* existed only at low salinity levels. Two former studies showed that along a salinity gradient, *Nitrosospira* was enriched at the high salinity sites while the genus *Nitrosomonas* tended to associate with low or middle salinity sites (Bernhard et al., 2005; Sahan and Muyzer, 2008). The contrasting results on the distribution of *Nitrosospira* might result from the other covarying factors, such as temperature and ammonia concentration; however, salinity was the only factor that drove changes in our study. Our results also indicate that *Nitrosomonas* might possess a broader adaption to the salinity levels compared to *Nitrosospira*. However, the mechanism on how they tolerate the salinity stress remains unknown.

It is suggested that denitrification is consistently decreased by soil salinization across coastal ecosystems (Zhou et al., 2017). In support of this, denitrifying activity declined with increased salinity from 0 to 30 ppt in this study. There are many mechanisms that may contribute to this phenomenon. For a laboratory incubation, salinity elevation might directly constrain the fitness of microbes and at the same time reduce the soil respiration (Wong et al., 2008) which could decrease the oxygen consumption thus inhibiting the anaerobic denitrifying process. The reduction of nitrification by salinity elevation can reduce the nitrate availability, which can also limit the denitrification (Giblin et al., 2010). In this study, the enhanced consumption of NO_x^- by salinity, which increased during the incubation, could be due to the promoted DNRA under high salinity conditions (Giblin et al., 2013; Marchant et al., 2014). Hence, our results potentially indicate the predominance of DNRA over denitrification in sediments that are subject to high salinities. On the other hand, the abundance of denitrifiers (*nirK* and *nosZ*) was also impacted by salinization, but inhibition only occurred at day 28, suggesting the tolerance of denitrifiers to sediment salinization at the beginning of the incubation. The decrease of the *nirK*/*nirS* ratio with increasing salinity indicated that the relative importance of *nirK* and *nirS* for reducing the nitrite might shift according to salinity levels and that *nirS*-bearing denitrifiers might be more tolerant to salinity elevation compared to those *nirK*. Moreover, the enhanced *nir*/*nosZ* ratio in

Table 1
The ANOSIM and PERMANOVA results showing the influence of salinity on nitrifying and denitrifying community compositions.

	ANOSIM		PERMANOVA	
	R	Significance	R^2	Significance
AOA	0.397	0.002**	0.409	0.010**
AOB	0.559	0.005**	0.556	0.002**
nirK	0.491	0.002**	0.479	0.003**
nirS	0.361	0.016*	0.355	0.025*
nosZ	0.361	0.005**	0.378	0.002**

Significance level is shown as *, $P < 0.05$; **, $P < 0.01$.

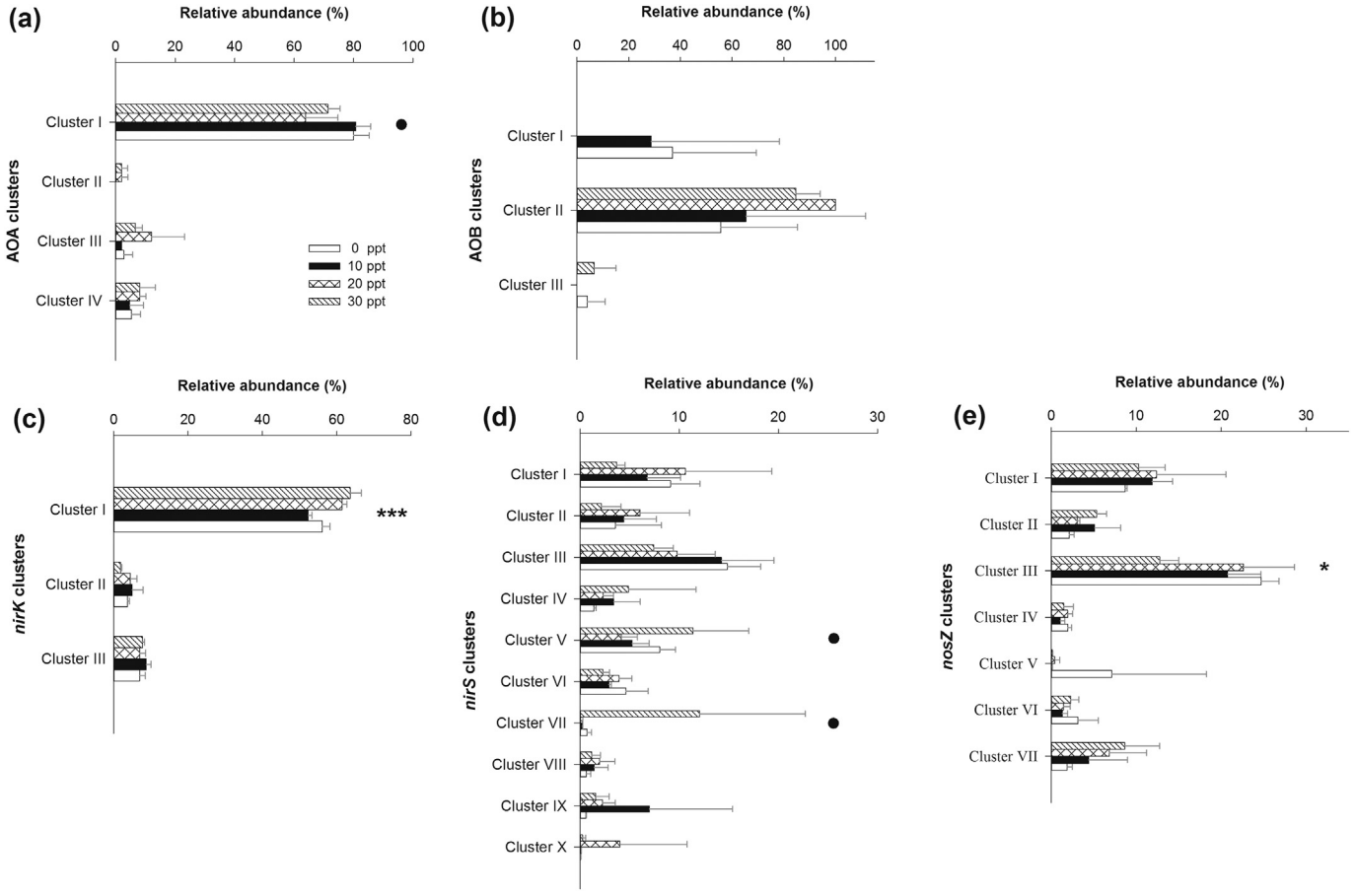

Fig. 5. The distribution of the clusters obtained from phylogenetic analysis and their relative abundances. The values are given as mean ± standard deviation (n = 3). Significance level is shown as ●, $P < 0.1$; *, $P < 0.05$; ***, $P < 0.001$.

30-ppt samples at day 28 illustrated that salinity elevation might rise the genetic potential for N_2O emissions since the higher *nir*/*nosZ* ratio can lead to an increased $N_2O/(N_2O + N_2)$ end-product ratio (García-Lledó et al., 2011).

A previous study found that salinity, in addition to other major factors, was associated with the dynamics of *nirK*, *nirS* and *nosZ* community composition in wetland sediments (Bañeras et al., 2012). The salinity was also found to correlate with *nosZ* community structures across eight tidal wetlands (Franklin et al., 2017). However, a study in the San Francisco Bay estuary revealed significant correlations between salinity and abundances of *nirK* and *nirS* but not their community structures (Mosier and Francis, 2010). Since these studies were conducted with samples *in situ*, the impact of salinity was coupled with other major factors such as vegetation, carbon source and metals. Hence our study does provide strong evidence that salinity can significantly shape the community structures of denitrifiers in the absence of other factors. Although we observed that the dominant *nirK* cluster I was significantly influenced by the salinity, little is known about this cluster. Similarly, the cluster V and VII of *nirS* were slightly influenced by the salinity, but they are also unknown taxa. The dominant cluster III of *nosZ* which might be associated with *Thiobacillus* was decreased by the high salinity (30 ppt). Mangrove contains high level of sulfides generated by sulfate reduction (Ferreira et al., 2007), and some species belonging to *Thiobacillus* can use the sulfur and oxidize sulfide to support their autotrophic growth (Friedrich et al., 2001). It has been recognized that sulfur oxidation can be coupled with nitrate reduction (Burgin and Hamilton, 2007); thus reduction of sulfide by oxidation might lead to the decrease of sulfur-driven nitrate reduction. Therefore, the inhibition of *Thiobacillus* by salinization might lead to the reduction of denitrification as we observed in this study.

In summary, we found the inhibiting effect of salinity elevation on the denitrifying activity and abundances of *nirK* and *nosZ*, while the *nirS* abundance remained stable. The abundance of AOB increased at intermediate salinity levels while AOA were less affected. However, a reduction in the consumption of NH_4^+ at high salinity levels might suggest the inhibition of nitrification by salinization. Therefore, the reduction of coupled nitrification-denitrification process might decrease the nitrogen removal, which might further lead to eutrophication and disturb the nitrogen balance in the environment. While there is limited information about the salinity and microbial community composition, this study provides strong evidence that salinity can significantly shape the community structure of both nitrifiers and denitrifiers. These results illustrate that salinity plays a vital role in regulating the nitrogen cycling in coastal areas, which may increase concern regarding threats of salinity intrusion associated with climate change and a rising sea level.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.03.102.

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